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## Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis

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### Abstract

A regulatory cascade, initiated during the syncytial stage of embryogenesis, culminates in the striped pattern of engrailed gene expression at the cellular blastoderm stage. The early regulatory genes, for example the pair-rule genes, are expressed transiently and as their products decay a distinct regulatory programme involving segment polarity genes takes over. This late programme maintains and perhaps modifies the striped pattern of engrailed expression through interactions that may involve cell communication.

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An early step in the generation of the *Drosophila* body pattern is the determination of the segmental subdivisions of the embryo. This occurs quite rapidly. By the cellular blastoderm stage (2–2.5 h after fertilization) the segmental outlines of the embryo, although not morphologically visible, are represented by the precise expression patterns of the segmentation (or segment polarity) genes, such as *engrailed*<sup>1,2</sup> and *wingless*<sup>3</sup>. The subsequent activity of genes such as these directs the morphological events leading to segmentation<sup>4,5</sup>.

The segmentation genes are expressed in their localized patterns as a consequence of an early regulatory cascade. Three gene classes (maternal genes<sup>6</sup>, and the zygotically acting gap and pair-rule genes<sup>4</sup>) act in sequence, each establishing the more refined spatial expression pattern of the next<sup>7–9</sup>. This takes place in a syncytial cytoplasm and is thought to rely on the diffusion and interaction of regulatory products encoded by these genes. The subdivision of the embryonic field by this cascade is rapid, occurring during the first thirteen nuclear divisions. After the thirteenth division, the nuclei are cellularized and the cellular blastoderm is formed.

One outcome of this regulatory hierarchy is the establishment of *engrailed* (*en*) expression at the cellular blastoderm stage in 14 single-cell-wide stripes transecting the antero-posterior axis of the embryo<sup>1,2,10,22</sup>. Previous work has shown that the activity of separate sets of pair-rule genes controls establishment of *en* expression in even- and in odd-numbered stripes<sup>11,12</sup>. The data presented here suggests further that the separate control of even- and odd-numbered stripes is reflected in the organization of the cis-regulatory sequences at *en*.

During the post-cellular blastoderm phase of development the expression pattern of *en* is maintained and modified<sup>1,10</sup>. It is unlikely that the pair-rule genes are involved in the post-blastoderm regulation of *en*, since the pair-rule genes characterized to date are only expressed transiently in their pair-rule blastoderm patterns<sup>13–16,31</sup>. Here we address the mechanism by which the *en* gene is regulated in later embryonic development.

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We find that the striped expression pattern of *en* during post-blastoderm development is controlled by a cis-regulatory programme distinct from that controlling the establishment of expression at the cellular blastoderm stage. Control of this late-acting programme is exercised through the activity of various segment polarity genes. The activity of at least one of the segment polarity genes (*wingless*) is not cell autonomous<sup>17,44</sup>. This leads us to a model for the control of post-blastoderm *en* gene expression involving cell interactions. We suggest that the late *en* regulatory programme allows for some plasticity in developmental programming.

## An *en-lacZ* fusion gene

As part of an ongoing dissection of the *en* regulatory region we have constructed transgenic fly lines expressing  $\beta$ -galactosidase under the control of portions of the *en* regulatory region. Our dissection of the cis-regulatory sequences at *en* is in its initial stages, but the expression pattern of one transgenic construct is particularly instructive. It accurately expresses discrete portions of the *en* pattern and represents one line of evidence that there are distinct early and late regulatory programmes that control the expression of *en* in stripes.

Figure 1 shows the DNA construct, P(*en/lac*), in which expression of a  $\beta$ -galactosidase ( $\beta$ -gal) - engrailed fusion protein is directed by the *en* promoter and flanking sequences. The fusion gene is in a vector allowing the establishment of stably transformed 'transgenic' flies through P-element mediated integration into the germ line<sup>18</sup>. In addition to the promoter and 5' flanking sequences, the construct includes 1099 base pairs (bp) of transcribed sequences from the *en* gene<sup>19</sup>, encompassing ~211 nucleotides of untranslated leader and the N-terminal 296 amino acids<sup>20</sup> spliced in frame to the *lacZ* gene encoding  $\beta$ -galactosidase ( $\beta$ -gal) activity. Therefore, the expression of this construct could reflect control both at the transcriptional and post-transcriptional levels. The signal ( $\beta$ -gal activity or antigen) produced by P(*en/lac*) is unstable, thereby allowing us to detect transitions that occur in *en* expression patterns. The instability could be due to the inclusion of *en* leader and coding sequences in the fusion, as a relatively stable  $\beta$ -gal signal is produced by a *ftz* promoter-*lacZ* fusion, which does not contain coding sequences from the *ftz* gene<sup>21</sup>.

## Early expression

Three independent P(*en/lac*) lines have been analysed, and all exhibit a similar pattern of ectodermal expression. Seven stripes of expression from P(*en/lac*) are apparent along the extending germband (Fig. 2*a*), a stage at which *en* is expressed in fourteen stripes. The seven stripes expressing P(*en/lac*) precisely align with the even-numbered *en* stripes (Fig. 2*b, c*). Within these stripes P(*en/lac*) is faithfully expressed since all cells expressing *en* are also expressing  $\beta$ -gal. On occasion, an extra  $\beta$ -gal-expressing cell is observed ventrally at the trailing edge of even-numbered *en* stripes (Fig. 2*b, c*). The significance of this occasional non-correspondence is unclear.

The establishment of P(*en/lac*) expression at even-numbered *en* stripes is first evident at the same stage as endogenous *en* expression—the cellular blastoderm/early gastrula (Fig. 2*d, e*). As endogenous *en* expression is established, the odd-numbered stripes initially lag behind the development of even-numbered stripes<sup>10,22</sup>. However, *en* is equivalently expressed in the odd- and even-numbered stripes by the time the germband is elongating (compare panels *d* and *b*, Fig. 2). In contrast, during germband elongation or even after the germband fully extends, no  $\beta$ -gal expression (enzyme or antigenic activity) is detected in positions corresponding to the odd-numbered *en* stripes (Fig. 2*a*).

To test whether the establishment of P(*en/lac*) expression is under the control of the same regulatory factors that control the establishment of even-numbered *en* stripes, we have analysed the expression of P(*en/lac*) in particular pair-rule mutants. Two pair-rule mutants known to

affect the expression of even-numbered *en* stripes, *fushi tarazu*<sup>11,12</sup> and *odd-skipped*<sup>12</sup>, have identical effects on the establishment of P(en/lac) expression and on endogenous *en* expression (data not shown).

We conclude that P(en/lac) expression is regulated faithfully at positions corresponding to even-numbered *en* stripes, and that this regulation involves the same trans-acting activities as the endogenous *en* gene. Therefore, the regulatory region present in P(en/lac) includes cis-acting sequences that direct the establishment of even-numbered *en* stripes. Presumably, the sequences required for expression in odd-numbered stripes are not present, or are functionally disrupted in this construct.

## Late expression

As development continues, there is a striking transition in the expression of P(en/lac). The changes in expression provide evidence that the expression of *en* stripes is separable into two temporally and mechanistically distinct programmes.

After the germband is fully extended, the expression of P(en/lac) in stripes corresponding to even-numbered *en* stripes begins to fade. (Note the spotty appearance of the stripes in Fig. 3a where the embryo is about 1.5h older than the stage exhibited in Fig. 2a.) At this late stage, individual cells located dorso-laterally at positions corresponding to odd-numbered *en* stripes begin to accumulate  $\beta$ -gal antigen (Fig. 3a). Over the next hour or two,  $\beta$ -gal staining is lost ventrally from even-numbered stripes, but intense expression is induced dorso-laterally at positions corresponding to both even- and odd-numbered *en* stripes (in the dorsal ridge, T1 through A7, and to some extent A8; Fig. 3b). The eleven dorso-lateral stripes expressed from P(en/lac) at this late developmental stage correspond cell-for-cell with *en* expressing cells (Fig. 3c, d).

As the germband retracts the P(en/lac) signal appears to extend ventrally, although it usually does not completely fill the *en*-expressing region. Often, the level of P(en/lac) expression on the ventral surface is higher in cells at the posterior edge of the *en* stripes, and lower in other cells of the *en* stripe (data not shown). In contrast, dorso-lateral expression of P(en/lac) remains uniformly coextensive with *en*-expressing cells. We conclude that the P(en/lac) construct contains at least some of the regulatory sequences necessary for position specific expression in ventro-lateral regions, whereas it contains all sequences necessary for dorso-lateral expression. The discrimination between dorso-lateral and ventro-lateral regions in the embryo is not unique to this construct. Recent data have shown that there is also a sharp ventral/dorsal distinction in neurogenic potential and in the mitotic division schedule<sup>23</sup> (V. Foe, personal communication).

At this later developmental stage, the expression of P(en/lac) in positions corresponding to odd-numbered *en* stripes is in striking contrast to the absence of detectable  $\beta$ -gal expression in these positions at early stages. Therefore, P(en/lac) responds later in development to regulatory signals that control expression in both even- and odd-numbered positions. These data show that two programmes of striped *en* expression are distinguishable by character and time of onset. The first programme results in the initiation of stripes at the cellular blastoderm, and involves the separate regulation of even- and odd-numbered stripes by pair-rule gene action. The second programme becomes evident after the germband has elongated, as initial expression fades and *de novo* expression becomes visible at both even- and odd-numbered positions. Since late P(en/lac) expression is not present in all cells that express the endogenous *en* gene (for example, ventral P(en/lac) stripes do not fill the *en* domain), it is unlikely that P(en/lac) expression is being regulated simply by the presence of endogenous *en* protein. Such a positive autoregulatory loop may be one of the components of late regulation, but *en* must also be responding to other signals. We next address the nature of these other inputs by investigating

the effects that particular segmentation genes have on the pattern of expression of the endogenous *en* gene.

## Regulation of late programme

It is unlikely that the pair-rule genes directly regulate the late programme of *en* expression, since products encoded by these genes are no longer detectable at this stage<sup>13–16,24,25,31</sup>. Analysing the *en* expression pattern in particular segment polarity mutants provides evidence that these genes are involved in regulating the second programme.

In *wg* null mutants *en* expression is established normally at the cellular blastoderm in fourteen roughly single-cell-wide stripes. This pattern remains wild-type through gastrulation and germband elongation, *en* stripes becoming 2–3 cells wide at this stage as a result of both cell movements and cell division. However, after the germband is fully extended, ectodermal *en* expression decays prematurely. Two developmental stages are shown in Fig. 4a, c, revealing the progress of the decay (summary, Fig. 6). The only ectodermal expression that remains is in the maxillary (Mx) stripe, a portion of the labial (La) stripe, and in a cluster of dorsally located cells in developing T1 (Fig. 4c). The effect of strong, but probably not null, alleles of *hh*, *arm* and *fu* are similar to the effects of the *wg* mutations, except that the *en* stripes do not disappear fully. Rather, a variable but small number of cells retain *en* expression. An example is shown for *hh*<sup>6N</sup> (Fig. 4d) (note the spotty and ‘thin’ appearance of *en* stripes). Weak alleles of *wg*, such as *wg*<sup>IL114ts</sup> grown at semi-permissive temperature, similarly leave some *en*-expressing cells unaffected (data not shown). This raises the possibility that the true null condition for *hh*, *fu* and *arm* (including removal of any maternal contribution), would result in the total loss of *en* expression as in *wg* nulls.

Some cell death occurs in these mutants during germband retraction<sup>26,27</sup>. Thus, it is possible that cell death leads to the observed loss of *en* signal. However, in *wg* mutants, there is no evidence of cell death at the stage when cells are in the process of losing *en* antigen (Fig. 4b). This suggests that the loss of *en* expression is an early effect in *wg* mutants and not secondary to cell death.

The results indicate that the segment polarity loci *wg*, *fu*, *hh* and *arm* act in some manner to reinforce or maintain *en* expression once it has been established. The segment polarity mutant *patched* (*ptc*)<sup>4</sup> has qualitatively different effects on *en* expression, providing further evidence for the existence of a distinct regulatory programme controlling *en* expression during post-blastoderm development.

In *patched* mutants, *en* expression is established normally. However, at full germband extension in *ptc* mutants, the program of *en* expression is altered as *en* is induced in cells lying a few cell diameters posterior to each normal *en* stripe (Fig. 5a). The cells that are inducing *en* lie near and/or within an abnormal furrow that develops in the ectodermal cell sheet (Fig. 5a, b, c). As development proceeds more cells express *en* (Fig. 5b). By the onset of germband retraction the ectopic expression forms an incomplete transverse stripe positioned eccentrically between each pair of normal *en* stripes (Fig. 5d; summary, Fig. 6).

The *ptc* mutant phenotype suggests that the novel *en* stripes have a functional consequence on development. The *ptc* mutants develop duplicated cuticular structures within each segment<sup>4</sup>. Among these duplicated structures are an anterior row of denticle hairs and a segment border, which are characteristic of *en*-expressing cells<sup>28</sup> (S. D., unpublished data; Fig. 5e,f). In a *ptc*, *en* double mutant, whereas other duplicated structures are still produced, those dependent on *en* activity are not produced. We conclude that the ectopic induction of *en* has a consequence on the final body pattern. Therefore, in *ptc* mutants particular cells that are initially specified

at the cellular blastoderm stage to be negative for *en* expression are reprogrammed later in development.

## **wg and ectopic induction**

The absence of the *ptc* product leads to a late programme of *en* expression in novel positions within a developing segment. Although pair-rule gene activity is unlikely to control late *en* expression in wild-type embryos, ectopic induction might in principle involve the same regulatory products that establish early *en* expression, such as *ftz* and *eve*, if their expression patterns are correspondingly altered in a *ptc* mutant. We consider this unlikely since we and others find no changes or increase in the lifetime of the *ftz*<sup>8</sup> and *eve*<sup>29</sup> patterns, nor evidence for renewed expression just before, or concomitant with, the induction of ectopic stripes. Furthermore, we can experimentally induce late expression of *ftz* in transgenic flies that express *ftz* from the inducible heat shock promoter<sup>30</sup> and such late *ftz* expression has no consequence on the late *en* pattern. The observations suggest that these pair-rule gene products are neither present nor competent to induce ectopic *en* expression at the time that ectopic *en* stripes appear in *ptc* mutants.

In fact, we find that ectopic induction of *en* in *ptc* mutants is dependent on *wg* activity. Specifically, in *wg, ptc* double mutants, *en* expression is established normally, but decays prematurely, as it does in *wg* single mutants. There is no subsequent ectopic induction as seen in *ptc* single mutants (data not shown).

The ectopic induction of *en* in positions where there was no previously detectable *en* accumulation raises the possibility that *wg* activity has the capacity to induce *en* expression *de novo*. Therefore, what we have been referring to as ‘maintenance’ of *en* expression by *wg*, and other segment polarity genes, may be a re-induction of *en* expression. This observation complements the conclusion drawn from the analysis of P(*en/lac*) expression, which suggested that the *en* program has a distinct, late regulatory component that is independent of the establishment of early expression.

## **Late control of en-lacZ**

Interestingly, the developmental stage at which segment polarity gene mutations affect *en* expression coincides with the transition between early and late *en* regulatory programmes as determined from the analysis of P(*en/lac*) expression (see above). The temporal link implies that the late programme of induction from P(*en/lac*) is due to the action of segment polarity genes. This has been tested by examining P(*en/lac*) expression in *wg* and *ptc* mutant embryos. In *wg* mutants, early P(*en/lac*) expression is identical to that in wild-type embryos, but the late programme does not initiate (data not shown). In *ptc* mutants, the early programme is also normal, but when the late programme is induced, P(*en/lac*) is expressed ectopically, as is the endogenous *en* gene (Fig. 3e,f). In summary, the effects of *wg* or *ptc* mutations on expression from P(*en/lac*) correlate with the effect of these mutations on endogenous *en* expression.

## **Cell communication**

The data show that there are two programmes regulating *en* gene expression in stripes during embryogenesis. The first regulatory programme involves the independent control of even- and odd-numbered *en* stripes by pair-rule gene action<sup>11,12,15</sup>, and may involve direct transcriptional regulation by some of the pair-rule products.

The second regulatory programme operates after cellularization, and involves segment polarity gene action. Recent molecular analysis of *wg*, coupled with the data presented here, suggests that cell interactions are involved in the late control of *en* striped expression.



The *wg* gene, like *en*, is expressed in stripes, but *wg* transcripts accumulate mainly in cells that are anteriorly adjacent to those that express *en*, and probably not in the cells that express *en* (ref. <sup>3</sup>, Fig. 6). Similarities noted between the predicted *Drosophila wg* protein and the product of the mammalian oncogene *int-1* have led to the suggestion that the *wg* product acts as a signal in cell communication<sup>32</sup>. This suggestion is consistent with genetic analysis showing that *wg* is not cell-autonomous<sup>17,33,44</sup>. Our results identify one set of target cells for *wg* activity—the neighbouring *en*-expressing cells (Fig. 6)—and we suggest that *en* gene expression may be a target of a signal that is initiated at the cell surface by the *wg* protein. Since *hh*, *arm* and *fu* have phenotypes similar to *wg* with respect to their effects on *en* expression, it is tempting to speculate that some of these genes may encode other components involved in the transduction of the *wg* signal. The existence of genes in the mouse that are homologous to *en*<sup>34</sup> and *wg*<sup>32</sup> suggests that the circuitry proposed here for the control of *en* by *wg* may be phylogenetically conserved.

In formal terms *ptc* activity is required to keep *en* expression repressed in particular cell rows within each developing segment. Interestingly, the derepression of *en* in a *ptc* mutant requires a signal from *wg*-expressing cells. Consequently, one function of *ptc* may be to prohibit, directly or indirectly, *wg* from inducing *en* ectopically (Fig. 6). Thus, *ptc* activity may impart a polarity on patterning within segments, since the induction of *en* is restricted to cells located posterior to the *wg* domain (Fig. 6).

Ectopic induction of *en* in *ptc* mutants occurs in cells which do not abut the normal *wg* domain. Either *wg* can act over several cell diameters, or there are other genetic interactions missing from the scheme. Indeed, Martinez-Arias and co-workers have found that the *wg* expression domain is expanded in *ptc* mutants and that this change precedes the induction of *en*<sup>35</sup>. Thus, the defects in a *ptc* mutant may lead to a cascade of compensatory changes, at least in part involving *wg*, that lead to the induction of *en*.

## Separate regulatory programmes

A teleological rationale for the existence of two discrete programmes for striped *en* expression can be offered. The observation that cells are respecified in *ptc* mutants suggests that there is still considerable plasticity in developmental programming at the cellular blastoderm. The later programme, involving the action of segment polarity genes, may represent an editing function that corrects subtle mistakes made in the establishment of *en* expression. In this scenario, the late programme is an important accessory programme that improves the fidelity of the system. In fact, although the establishment of *en* expression at blastoderm is remarkably precise—>95% of the cells in even-numbered *en* stripes map precisely to the anterior-most cells of *ftz* stripes<sup>36,37</sup>—the remaining 2–5% of cells may represent true ‘mistakes’<sup>37</sup>, where *en* expression is initiated in cells internal to the anterior edge of the *ftz* stripe. Perhaps these mistakes are corrected during the post-blastoderm phase of development. In addition, this editing function may be what governs the classical ‘regulative response’, in which adjustments are made in body pattern in response to physical perturbations<sup>38,39</sup>.

A second possibility, not incompatible with the first, is that the existence of a separate early programme is an evolutionary adaptation for the very rapid embryonic development observed in *Drosophila*. The early programme, which establishes striped *en* expression at cellular blastoderm, represents the successful conclusion of a regulatory cascade that subdivides the embryonic field very rapidly<sup>9</sup> and operates largely in a syncytium. Such a rapid and syncytial developmental plan is rather peculiar. Perhaps the late regulatory programme, involving cell interactions, is sufficient to achieve the gradual and sequential subdivision of the germband seen in most organisms. In this scenario, the late programme would be phylogenetically conserved, and the rapid cascade of regulatory interactions that culminates in the early program

would be an accessory programme used by *Drosophila* and its relatives to rapidly establish spatial subdivisions.

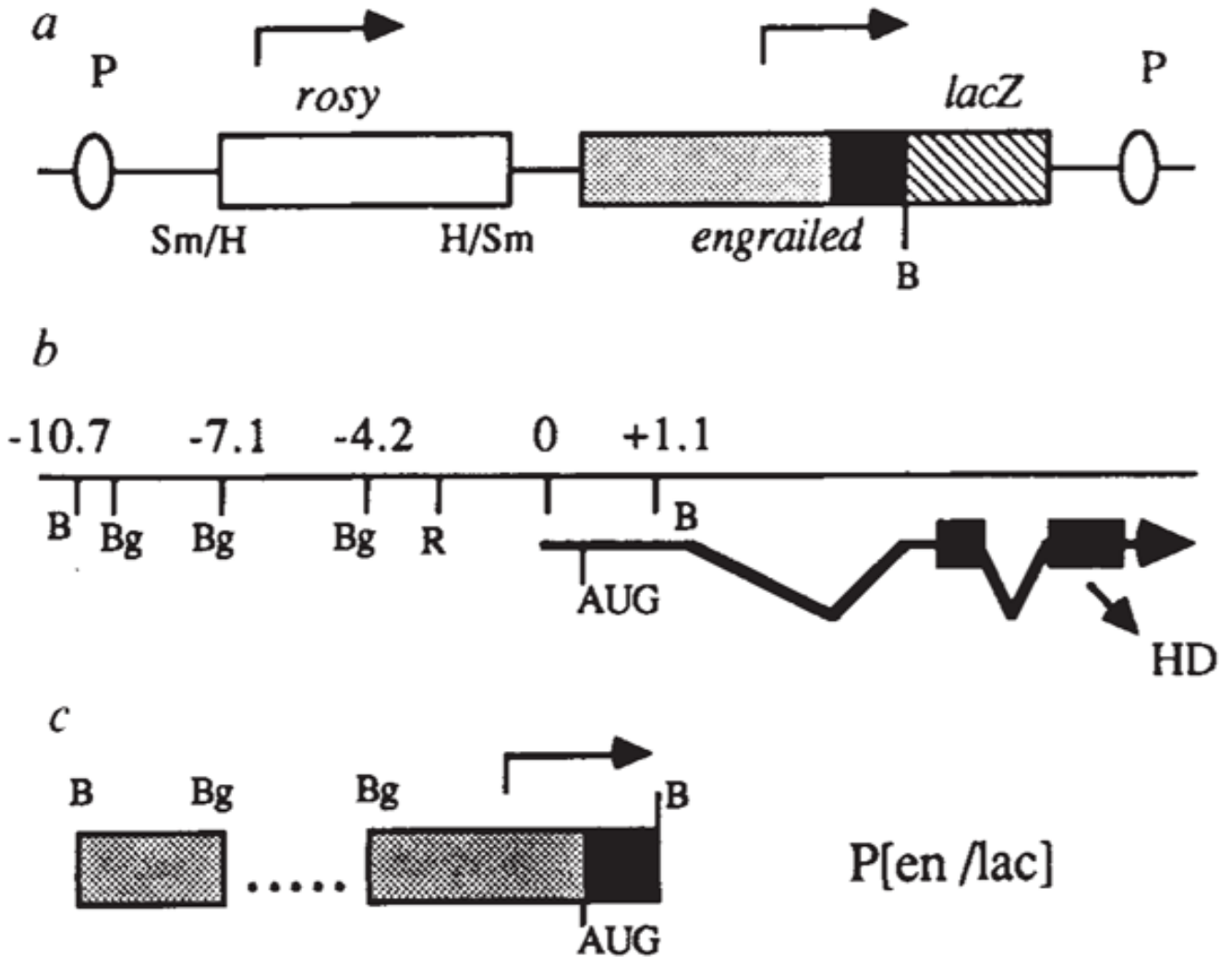
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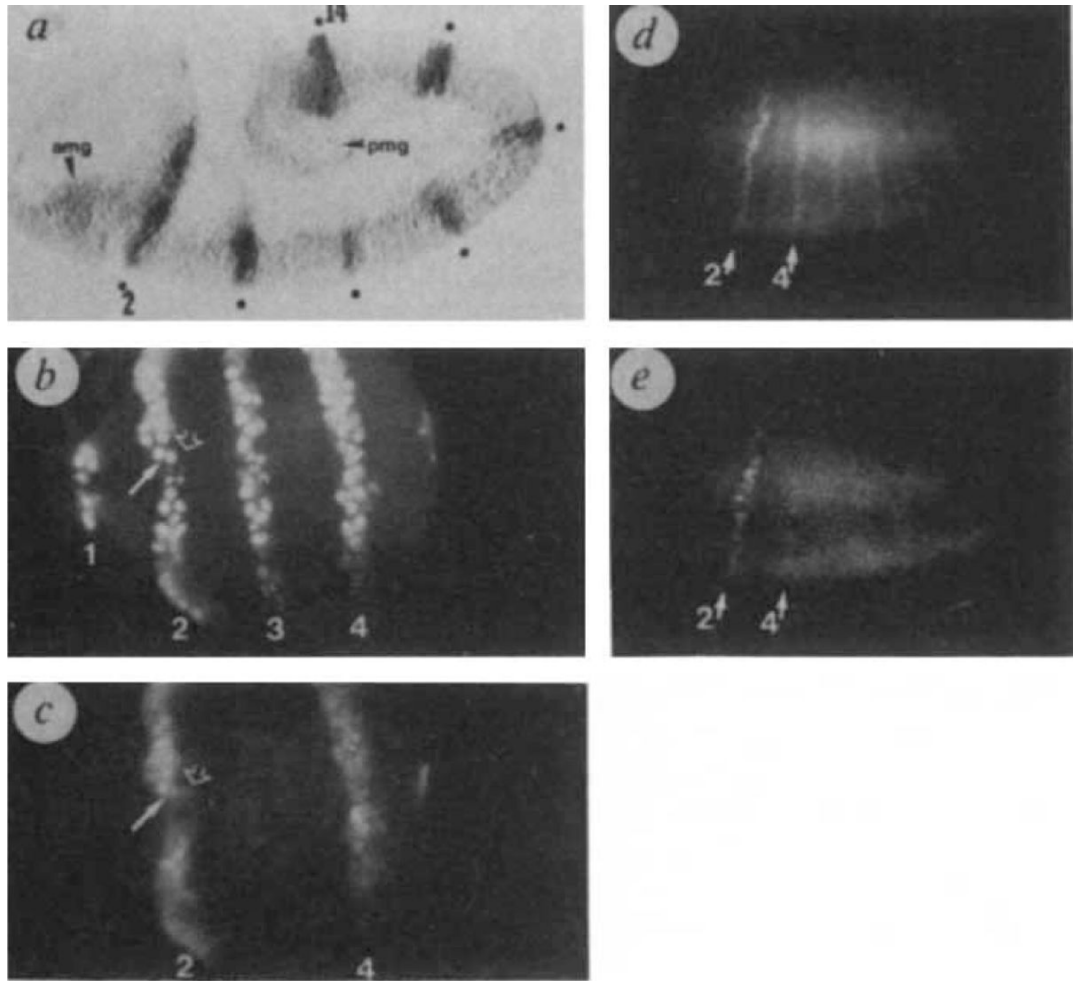




**Fig. 1.**

Construction of an *en-lacZ* fusion gene (not to scale), *a*, schematic diagram of P(*en/lac*) indicating sites for P-mediated integration (P); the *rosy* gene (open box); upstream and non-coding (stippled) and coding (filled) regions from *engrailed*; and *lacZ* coding sequences (hatched). An SV40 polyadenylation signal is at the end of *lacZ*. *b*, Map of *en* genomic region showing particular restriction site landmarks, the major transcription unit with start site (0) and presumed translation start site (AUG) and the portions of exons two and three (solid boxes) encoding the homoeodomain (HD)<sup>19,20,40</sup>. *c*, Specific genomic *en* regions used in constructing P(*en/lac*) (aligned with *b*). Dots represent the deletion of sequences between coordinates -4.2 and -7.1. Restriction sites: B, *Bam*HI; Bg, *Bg*III; H, *Hind*III; R, *Eco*RI; Sm, *Sma*I; symbol (/), joined by blunt-end ligation. Arrows indicate the direction of transcription.

**Methods.** Transgenic lines were established<sup>18</sup> using ‘wings-clipped’ helper, and an *Sgs-4<sup>Ber-1</sup>*, *cn*; *ry*<sup>506</sup> host. Lines were established from three independent G<sub>0</sub> adults; the insertion mapped to chromosome II in one line and to chromosome III in the other two.

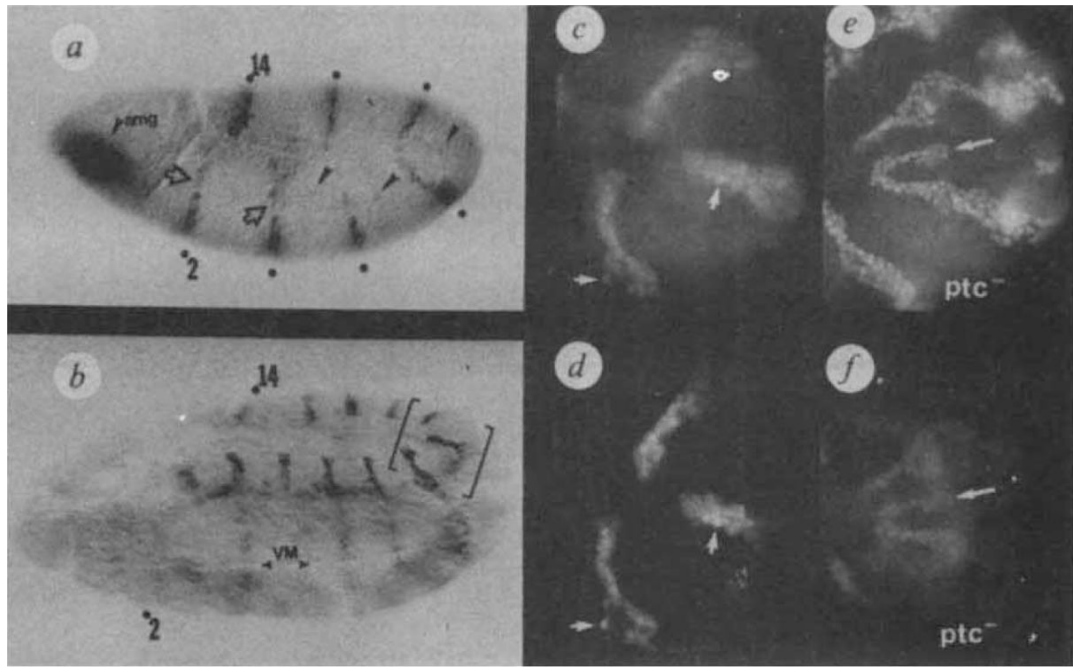


**Fig. 2.**

Antibody-staining of P(en/lac) transgenic embryos show that regulatory sequences controlling establishment of even-numbered *en* stripes are functionally distinct from those controlling establishment of odd-numbered stripes. Anterior left and ventral down in all panels, unless indicated otherwise, *a*, P(en/lac) transformant stained with rabbit anti- $\beta$ -galactosidase antibody to visualize the expression from P(en/lac), 3.5–4h after egg laying (AEL). Seven ectodermal stripes are evident (indicated by dots), corresponding to *en* stripes 2, 4, 6, 8, 10, 12 and 14, which mark the posterior portions of the maxillary, first and third thoracic, and second, fourth, sixth and eighth abdominal primordia. Variable expression seen in the anterior (amg) and posterior (pmg) midgut primordia is not part of the normal *en* expression program. This expression may be due to sequences from *en*, or may be spurious, since expression from similar vectors in mesodermal and gut primordia has been reported<sup>41</sup>. *b*, *c*: Magnified (ventral) view of a doubly-labelled embryo (mouse anti-*en* and rabbit anti- $\beta$ -gal) reveals expression of endogenous *en* (*b*) and of P(en/lac) (*c*), 4.5 h AEL. The stripes of *en* expression are 1(Mn), 2 (Max), 3(La) and 4(T1). Expression of  $\beta$ -gal is coincident with even-numbered *en* stripes 2 (Max) and 4(T1); arrows indicate the same cells in *b* and *c*. There is no detectable  $\beta$ -gal expression at positions corresponding to odd-numbered *en* stripes. In even-numbered stripes there is virtually one-to-one correspondence between  $\beta$ -gal and *en* expressing cells. The occasional cell containing low levels of  $\beta$ -gal antigen but no detectable *en* is indicated (open arrow). The  $\beta$ -gal signal is not as discrete as the *en* signal since the en- $\beta$ -gal fusion protein is not restricted to the nucleus, *d*, *e*: Embryos were doubly-labelled for *en* expression (*d*) and  $\beta$ -

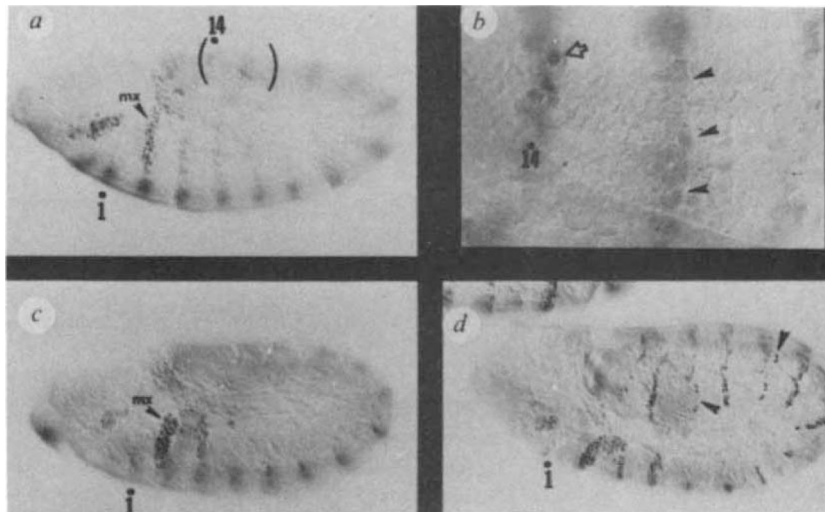
gal expression (*e*) at the onset of gastrulation, 3 h AEL. Expression from P(en/lac) is induced at about the same developmental stage as *en*, although there may be a slight lag in induction or accumulation. Arrows point to co-expression in 2(Max), and in 4(T1), where  $\beta$ -gal is just detectable.

**Methods.** Preparation of embryos for immunocytochemistry was as in refs <sup>10, 12</sup>. Rabbit anti- $\beta$ -gal was from Cappel, rabbit anti-*en*, (ref. <sup>10</sup>); mouse monoclonal anti-*en* (and *inv*), a gift of K. Coleman, C. Goodman and T. Kornberg. Final magnification was  $\times 130$ , except for *b* and *c* which were  $\times 520$ .



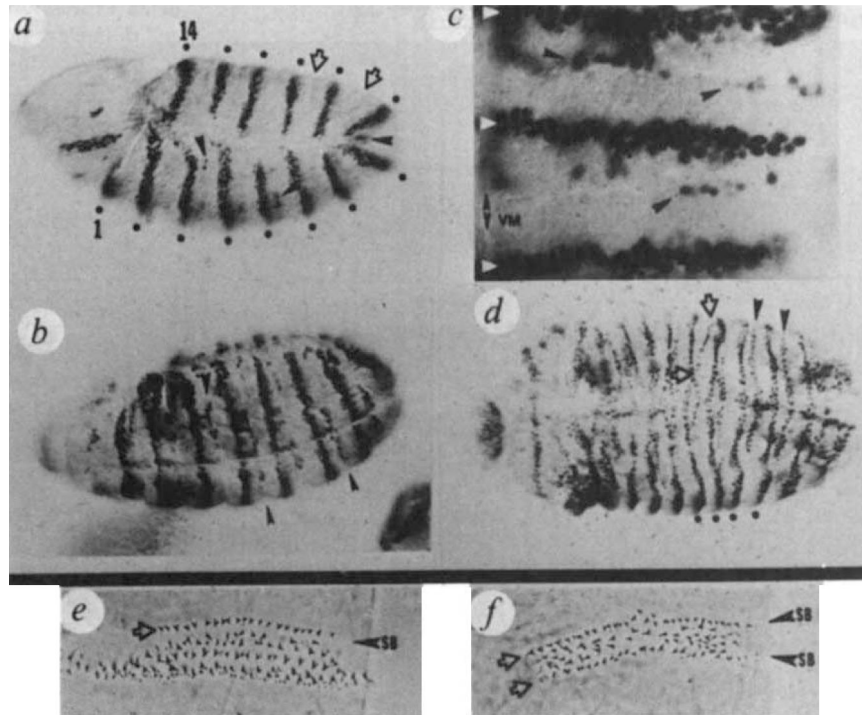
**Fig. 3.**

P(en/lac) uncovers a distinct late program of control over *en*, which is regulated by segment polarity genes, *a*, staining for  $\beta$ -gal, 5 h AEL, anterior left and ventral down ( $\times 130$ ). The seven  $\beta$ -gal stripes (dots) are beginning to fade. Note the spotty appearance laterally (open arrows). As development proceeds this signal fades ventrally. In addition, note that some cells at dorso-lateral positions are beginning to accumulate  $\beta$ -gal signal (solid arrows). The signal in the dorsal ectoderm will be heavily induced during subsequent stages. Though restricted to dorsal regions, the position of this signal corresponds accurately to even- and odd-numbered *en* stripes, *b*, Staining for  $\beta$ -gal at the onset of germband retraction, 7–8 h AEL, ventro-lateral view ( $\times 130$ ). Expression of P(en/lac) in ventral areas is low, but expression at dorso-lateral positions is quite high. Expression in both ventral and dorsal positions occurs in the posterior portions of 11 segment primordia: Labial, T1–T3, and A1–A7. Significant expression of P(en/lac) in Mandibular and Maxillary primordia and the terminalia during this late programme is usually not observed. Expression in dorsal-most A8 is sometimes observed. Bracketed area is magnified (from a different embryo) in *c* and *d*. VM, ventral midline. *c*, *d*: Magnified view ( $\times 520$ ) of a doubly-labelled embryo at the turn of the germband (A2, A3 and A4 region) showing one to one correspondence between *en* (*c*) and  $\beta$ -gal (*d*) expressing cells, *e*, *f*: Same as *c*, *d* but in *ptc* mutant background, with arrow showing that P(en/lac) is ectopically induced (*f*) as is the endogenous *en* gene (*e*).



**Fig. 4.**

Premature decay of *en* expression in *wg* and *hh* mutants. Embryos, anterior left and ventral down, were stained with anti-*en*-antibody, *a*, Mutant *wg*<sup>cx4</sup>: 4–5 h AEL (×130). Most *en* stripes are faint, but still visible above background. The Max and La stripes are affected least. Similar results have been obtained in *wg*<sup>L5</sup>, *wg*<sup>IG22</sup>, and also in *wg*<sup>IL114ts</sup> when aged at non-permissive temperatures (25–28 °C). In wild-type embryos *en* antigen can be visualized in cells until the onset of cuticular differentiation, 12–14h AEL<sup>10</sup>. *b*, Mutant *wg*<sup>cx4</sup>: magnified (×520) view (in a different embryo) of bracketed region shown in (*a*) as signal from *en* expression decays. Particular *en* stripes lose signal at varying times; thus, these two adjacent *en* stripes clearly show that the decay in *en* expression occurs while cells are still part of the ectodermal cell sheet (arrowhead, decaying signal), *c*, Mutant *wg*<sup>cx4</sup>: 6.5 h AEL (×130). The remaining *en* signal is lost from ectodermal cells, except in Max and La regions. The blurred ventral signal is due to *en* expression in cells (out of this focal plane) of the developing central nervous system, *d*, *hh*<sup>6N</sup>: 6 h AEL (×130). Note the spotty and thin appearance of *en* stripes. Similar results are found for *arm*<sup>xk22</sup>, *fu*<sup>113</sup>, *fu*<sup>113/fu</sup><sup>94</sup>, *hh*<sup>6n/hh</sup><sup>9k</sup>, and in *wg*<sup>IL114ts</sup> (raised at 16–18 °C). The segment polarity mutants *gooseberry* and *cubitus interruptus*<sup>D</sup> have not been studied.

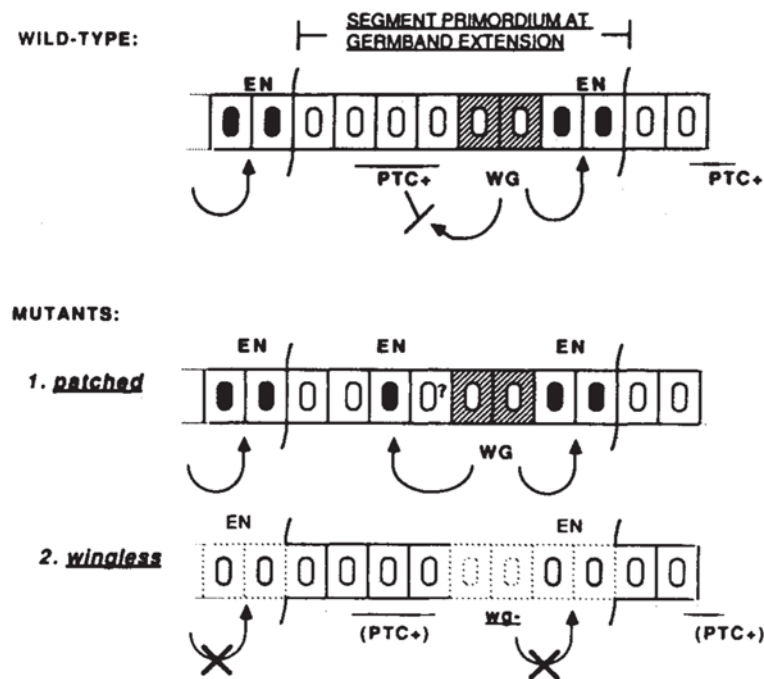
**Fig. 5.**

Reprogramming of cells to express *en* in *patched* mutants. Mutant embryos *ptc*<sup>IN108</sup> were stained with anti-*en*-antibody, *a*, Germband extended embryo (4.5 h AEL), anterior left and ventral down ( $\times 130$ ). The normal 14 *en* stripes, (established at the cellular blastoderm stage) are now two to three cells wide and are indicated by dots. The patches and stripe of *en*-expressing cells anterior to stripe 1 resemble wild-type. Cells newly accumulating *en* antigen are indicated (arrowheads). The stage at which ectopic expression is first detectable cannot be due to leakiness of the *ptc* allele because the same delay is observed in *ptc*<sup>IN108</sup> homozygotes as in the deficiency/*ptc*<sup>IN108</sup> heteroallelic combination. A furrow in the ectodermal cell sheet is becoming visible between each pair of *en* stripes at the approximate position of ectopic induction (open arrows). The asterisk marks a cell group that induces *en* at this stage in wild-type also, *b*, Ventral view, 6 h AEL ( $\times 130$ ). Eight *en* stripes are visible. More cells now express *en* between each pair of normal stripes, giving the appearance of an eccentrically positioned, incomplete stripe (arrowheads), *c*, Magnified ( $\times 520$ ) ventro-lateral view (6h AEL) showing three normal *en* stripes (white arrowheads) and cells that have induced *en*, located near and often within the deep furrows, *d*, Germband retracted (9 h AEL) anterior left ventral view ( $\times 130$ ). A few normal stripes (dots) and the intervening ectopic stripes are indicated (arrowheads). A given ectopic stripe has a wavy appearance, fusing along part of its length alternately with a posteriorly located *en* stripe, and then ventro-laterally with an anteriorly located stripe (open arrows). This fusion may be the result of shifts in cell position during germband retraction. Such fusions and reorganization of *en* stripes are frequently seen at late developmental stages in many of the segmentation mutants, *e*, Wild-type fourth abdominal denticle belt with border (SB) between third (upper) and fourth (lower) segment indicated. *f*, Fourth abdominal denticle belt in mutant *ptc*<sup>IN108</sup>/*Df*(2R)*ptc* showing duplication of segment border (SB), and anterior row of denticles (open arrows). In *ptc*<sup>6p43</sup>, *en*<sup>IM99</sup> double mutants (raised at 18–20 °C) both normal and duplicate anterior row denticles do not appear, showing dependence on *en* function.

**Methods.** Stocks were obtained from C. Nusslein-Volhard and E. Wieschaus, from N. Baker (*wg*<sup>cx4</sup>), and J. Hooper and M. Scott (*Df*(2R)*ptc*44C–E). Crosses were performed in standard



fashion, at 25 °C on molasses-cornmeal-agar. Embryos were prepared for immunocytochemical detection of en protein by affinity-purified rabbit anti-en antibody<sup>10,12</sup>. Cuticles were prepared as in ref. <sup>42</sup>.



**Fig. 6.**

Interactions regulating *en* during post-blastoderm development. Wild-type: The segment primordium at germband extension stage (anterior to left) is represented as eight cells wide—the roughly four-cell wide primordia having expanded due to a post-cellular blastoderm cell division and some interdigitation of cells. Several domains within each primordium appear to exist, each 'marked' by the expression of, or by the requirement for the activity of, particular genes. Cells expressing *en* and *wg* account for two of the domains: the *en* domain comprises the last two cells of the primordium<sup>4–6</sup> (blackened nuclei); the anteriorly adjacent two cells constitute the *wg* domain<sup>3</sup> (hatching). The analysis of *ptc* mutations suggests that the remaining cells of the primordium are divided into at least two domains: those that ectopically induce *en* in the absence of *ptc* activity, and those that do not. The placement of the *ptc*-requiring domain is based on the position of ectopic *en* expression in *ptc* mutants. It has been proposed that the activity of another gene, *naked*, is required for the anterior-most cells of the primordium<sup>35</sup>. Each of the domains is presumably established at the cellular blastoderm stage by the action of pair-rule segmentation genes, as has been shown for *en*<sup>11,12</sup> and *wg* domains<sup>43</sup>. The establishment of *en* expression constitutes the early regulatory programme of *en*. The second regulatory programme acts during post-blastoderm development as *wg*-expressing cells induce (or maintain) *en* expression in posteriorly located cells (arrow). Data presented here do not rule out other interactions. Mutants: (1) In the absence of *ptc* activity, *en* is induced in cells anterior to the *wg* domain. The induction requires *wg* (arrow to anterior), as no induction is detected in the absence of *wg* activity. Thus, in wild-type, *wg* induces *en* posterior to the *wg* domain, but is inhibited, directly or indirectly, from doing so anteriorly by *ptc* activity (blocking arrow shown in 'wild-type' panel). A cell (marked '?') lies between the *wg* domain and the cell ectopically inducing *en* (see text). (2) the *wingless* mutation leads to the premature loss of *en* expression (stippled nuclei), as a result of the loss of a morpho-genetic signal from *wg* expressing cells, perhaps the *wg* product itself. The *hh*, *fu* and/or *arm* products may play some role in the transduction of this signal. Cuticle pattern normally produced by *en* and *wg*-dependent cell types is missing<sup>3,4</sup> either due to cell death or transformation of fate (dashed cell outlines). It is not known if the *wg* mutation affects cells located anteriorly to the *wg* domain.